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Metabolism and macromolecular interaction of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in cultured explants and epithelial cells of human buccal mucosa

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Metabolism and macromolecular interaction of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were studied in human buccal mucosa *in vitro*. Microautoradiographic analysis of [5-^{3}H]NNK-exposed explant cultures demonstrated a uniform distribution of bound radioactivity in the mucosal epithelium, without significant binding in the underlying connective tissue. The metabolism of [5-^{3}H]NNK at concentrations of both 6 and 100 μM resulted in seven identified metabolites in both explant and epithelial cell cultures. Formation of 4-(methylnitrosamino)-1-(3-pyridyl)butan-1-ol by carbonyl reduction of NNK accounted for almost 95% of the total metabolism, whereas the proportions of other metabolites obtained by α-carbon hydroxylation and pyridine *N*-oxidation reactions varied from undetectable levels to ~2% in both experimental systems. A positive correlation between concentration and the metabolic route associated with the formation of DNA methylating intermediates (α-hydroxylation at the *N*-methylene carbon) was found, i.e. when the concentration of NNK was raised from 6 to 100 μM, keto-acid formation which in part reflects DNA methylation was increased preferentially over ketoalcohol production, an index of DNA pyridyloxobutylation. Both the total rate of NNK metabolism and the amount of protein adducts were higher in cells from primary cultures up to the third passage than in explants cultured for 1 day. Between 10^{-9} to 10^{-4} M, neither NNK nor its precursor alkaloid nicotine affected the colony forming efficiency of normal and tumorous buccal epithelial cells, although at 10^{-3} M each agent inhibited this function. Taken together, the results demonstrate the capability of human buccal mucosal epithelium to metabolize NNK by three major pathways, including those involved in the formation of adducts with cellular macromolecules.

Introduction

Tobacco-specific *N*-nitrosamines are present at levels of 1–100 μg/g of tobacco products and are possible causative agents for oral cancer (1–6). During chewing and snuff dipping, the formation of additional amounts of *N*-nitrosamines from tobacco alkaloids, e.g. nicotine and nitrosating agents, has also been suggested (6,7). Yeast infection of oral epithelium may also result in *N*-nitrosamine formation (8). During the metabolism of procarcinogens like the tabacco-specific *N*-nitrosamines, reactive intermediates are formed, some of which can react with biological macromolecules to produce covalently bound adducts (1,2). The most prevalent tobacco-specific *N*-nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) and *N'*-nitrosonornicotine (NNN) are strong carcinogens in laboratory animals, and cause the formation of tumors in various tissues (1,2,9). Application of a mixture of NNK and NNN on to the oral mucosa has been shown to produce tumors at or near the treatment site in F344 rats (10). The dose used in these animals approximates life time exposure levels for some snuff dippers (2). Comparative metabolism and macromolecular interaction studies with NNK and NNN in cultured rat oral mucosa demonstrated detectable levels of DNA adducts from NNK only, underlying a role for NNK in tobacco-related carcinogenesis in this tissue (11,12). Moreover, administration of NNK or NNN to the cheek pouch of Syrian golden hamsters induced tumors only from the NNK treatment (13).

Previously, NNK and NNN were shown to undergo metabolism in explant cultures from human aerodigestive tract, including the oral mucosa (14). However, these studies were limited to few specimens cultured at conditions optimized for tissues other than oral mucosa and, moreover, identified only two metabolities of NNK (14). As described below, more recent studies have demonstrated additional metabolities of NNK in various experimental systems through the use of high performance liquid chromatography and identification based on cochromatography with reference compounds (1,15).

Metabolism of NNK occurs by carbonyl reduction, pyridine *N*-oxidation and α-hydroxylation (1) (Figure 1). Carbonyl reduction of NNK leads to the formation of an *N*-nitroso alcohol 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is a major metabolite in many tissues and also a potent carcinogen (16–18). In contrast, pyridine *N*-oxidation results in the formation of 4-(*N*-methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone, which is less DNA damaging and a weaker carcinogen than NNK (15,16). α-Hydroxylation at the *N*-methylene carbon would result in the electrophilic intermediate methyl diazohydroxide, which methylates protein and nucleic acids. A similar hydroxylation at the *N*-methyl carbon of NNK is believed to yield the pyridyloxobutyl diazohydroxide (19). This intermediate or the carbonium ion derived from it reacts with protein, e.g. globin, and DNA to form pyridyloxobutyl adducts (20). Considering that the major metabolite NNAL may also be metabolized by α-hydroxylation (1,15), the summation of products (denoted 5, 6, 7, 8 in Figure 1) provides an 'index of activation' of NNK and NNAL by α-hydroxylation reactions. As shown in Figure 1, NNAL can also be converted to its *N*-oxide, although this pathway is considered as a deactivation mechanism (15,16).

We have recently developed conditions for obtaining epithelial

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN, *N'*-nitrosonornicotine; BEX, a medium used for buccal explant culture; CFE, colony forming efficiency.

cell cultures of human buccal mucosa, either from explant outgrowths or from growth of cells isolated by enzymatic dissociation of the epithelium (21,22). Explants maintained in a serum-free and growth-factor supplemented MCDB 153 medium that contains 0.1 mM Ca^{2+} are viable and produce several epithelial outgrowths for up to 2 months (21). However, the suprabasal epithelium exfoliates from the buccal explants within 1–3 days under these and other culture conditions developed for various human tissues (21, unpublished data), implying a need for the development of conditions that preserve the structure of this tissue. In this regard, a prolonged maintenance of a normal tissue architecture in murine oral explants is supported by elevation of the Ca^{2+} concentration to the mM range (23).

To investigate metabolism and macromolecular interactions of NNK in human buccal mucosa, several specific aims were addressed in the present study. These included the establishment of conditions that allow maintenance of the morphology of tissue specimens in short-term explant culture, and moreover, the subsequent use of this system and epithelial cell cultures to study the metabolism of [5-^{3}H]NNK. In order to also study the macromolecular interaction of NNK in this tissue, high-resolution microautoradiographic analysis of [5-^{3}H]NNK-exposed explants was performed. Formation of protein adducts was also quantitated in both explants and cells by measurement of radioactivity covalently associated with protein. Finally, the effects of NNK and its precursor alkaloid nicotine on the colony forming efficiency (CFE) of both normal cells and a buccal carcinoma cell line (SqCC/Y1) were determined.

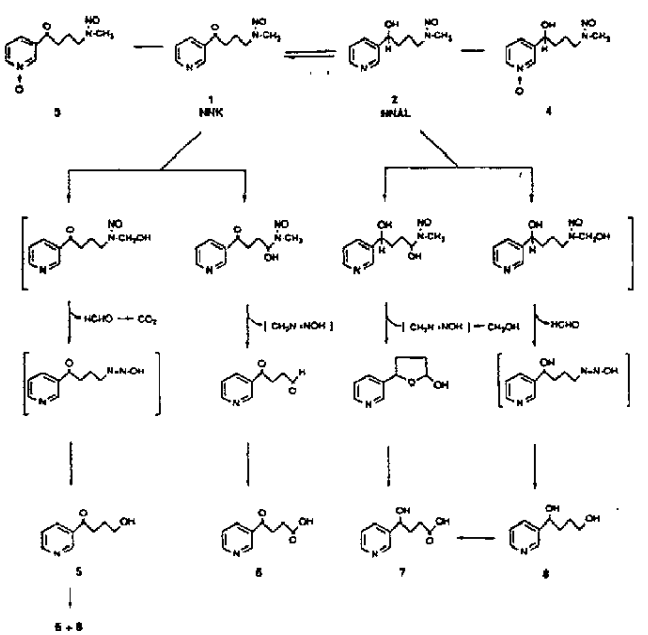

Fig. 1. Metabolic pathways of NNK (adapted from reference 1).
Compounds: 1. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone;
2. 4-(methylnitrosamino)-1-(3-pyridyl)butan-1-ol;
3. 1-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone;
4. 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)butan-1-ol;
5. 4-hydroxyl-1-(3-pyridyl)-1-butanone; 6. 4-oxo-4-(3-pyridyl)butyric acid;
7. 4-hydroxy-4-(3-pyridyl)butyric acid; 8. 4-hydroxy-4-(3-pyridyl)-1-butanol.

Materials and methods

Chemicals

[5-^{3}H]NNK (NNK with the tritium label in the 5-position of the pyridine ring, 1.15 Ci/mmol, purity >99.5%), and unlabelled NNK (purity >99%) were purchased from Chemsyn Science Laboratories, Lenexa, KS. The metabolites of NNK (Figure 1) were synthesized as previously described (24,25). MCDB 153 medium was from NordVacc Biotechniques AB (Huddinge, Sweden) or prepared from various stock solutions (22). Trypsin, gentamicin, penicillin-streptomycin and fungizone were purchased from Gibco, Labdesign AB, Lidingö, Sweden. Other reagents used were of analytical grade.

Explant culture

Human buccal tissue specimens, without evidence of pathological changes, were obtained from donors undergoing maxillofacial reconstructive surgery, or at autopsy performed within 6 h after death (21). Explants (2–5 mm^2) with the epithelial side up were cultured in Petri dishes placed in a rocking chamber under 50% O_2, 45% N_2 and 5% CO_2 (21), either on top of a gelatin-sponge or directly on the scratched surface of the dish. A serum-free buccal epithelial growth medium BEG-1 (21) with the Ca^{2+} concentration elevated 10-fold, i.e. to 1 mM, was used for short-term cultures of explants (up to 5 days) and was termed BEX, as for buccal explants.

Cell culture

Oral tissue, obtained as above, was incubated in 0.17% trypsin in HBS at 4°C for 18–24 h, to separate the epithelium from the underlying tissue (22). The epithelium was then scraped to remove additional cells, and the resulting suspension of single cells and small aggregates was then collected, rinsed, pelleted and resuspended in growth medium as described (22). Stock cultures of epithelial cells were initiated in a medium termed EMA (22), that is based on MCDB 153 with the Ca^{2+}-concentration adjusted to 0.1 mM, containing insulin (0.12 U/ml), phosphoethanolamine (0.1 mM), ethanolamine (0.1 mM), hydrocortisone (0.5 μM), epidermal growth factor (1.64 nM), gentamicin (50 μg/ml) and bovine pituitary extract (20 μg/ml) (22). Upon reaching ~75% confluency, the primary cultures were dissociated using trypsin (0.25%), ethyleneglycol-bis-(β-aminoethyl ether) *N*, *N*, *N'*, *N'*-tetraacetic acid (0.02%) and polyvinyl pyrrolidone (1%) in HBS and the cells were pelleted, resuspended in fresh growth medium, inoculated and passaged on fibronectin/collagen-coated dishes at 5×10^3 cells/cm^2 (22). A buccal carcinoma cell line (SqCC/Y1) was grown and passaged using the similar culture conditions and methods as for normal cells, except that regular non-coated dishes were used (22).

Colony forming efficiency

The normal and tumorous cells were seeded at 250 and 50 cells/cm^2 respectively, in 60 mm dishes and incubated for 24 h in EMA prior to treatment. Subsequently, fresh EMA with the respective agent (NNK or nicotine) was added at the indicated concentrations, and the cells were incubated for 8 days before fixation in 10% formalin and staining with 0.1% aqueous crystal violet. The mean colony forming efficiency was determined from duplicate dishes based on colonies each containing at least 16 cells. The colony forming ability was ~10 and 30% of the number of cells seeded from normal and tumorous cells respectively. The variation among replicate dishes of non-exposed controls was 5% or less in both cell types. Student's *t*-test was used to evaluate differences between control and treated cells.

Metabolism of NNK

Explants were initially incubated in BEX for up to 4 h and then for another 24 h in fresh BEX containing [5-^{3}H]NNK either at 6 μM (818 mCi/mmol) or at 100 μM (98 mCi/mmol). Epithelial cells in primary culture or in passages 1–3 were incubated in BEG-1 for 1 h with either of these concentrations of [5-^{3}H]NNK. After the incubations, the explants or cells and media were each separated and collected, and samples were frozen at −20°C until analysis. Medium was analyzed for NNK metabolites on a Waters HPLC system 501 equipped with a μBondapak C_{18} column 3.9 × 30 cm (Waters Associates, Millipore, Stockholm, Sweden) using sodium acetate buffers and non-radioactive marker metabolites (marked 1–7 in Figure 1) as internal UV standards as previously described (16). Radioactivity was determined in 1 ml fractions by the addition of 5 ml of Instagel (Packard Instrument AB, Bandhagen, Sweden). More than 90% of [5-^{3}H]NNK added to the culture medium was recovered in the final analysis. Explants that had been treated with boiling water for 5 min were used as negative controls, and did not exhibit detectable metabolic activity.

Formation of protein adducts

Explants or cells that had been exposed to [5-^{3}H]NNK were washed three times with Tris–HCl buffer (pH 7.2), homogenized in 1 ml of this buffer and then sonicated three times for 1 min with intermittent cooling of the samples for 30 s on ice. The protein was precipitated with 10% trichloroacetic acid and pelleted by centrifugation at 1200 *g*. The pellet was suspended and repelleted once with

10% trichloroacetic acid, and the procedure was repeated at least five times or more with methanol until no radioactivity over background could be detected in the supernatant. The precipitate was subsequently dissolved in 1 ml of 1 M NaOH overnight at 37°C. The samples were aliquoted to assay covalently bound radioactivity by scintillation counting and to measure protein content according to Bradford (26). Student's *t*-test was used to evaluate differences between the respective treatments and experimental systems.

Microautoradiographic localization of covalently bound NNK metabolites in explant cultures

The cultured tissues were fixed in buffered 4% formaldehyde solution (pH 7.0), serially dehydrated in ethanol, embedded in Historesin™ (LKB-Produkter AB, Bromma, Sweden) and 2 μm sections were cut and collected on glass slides (27). The slides were dipped in Kodak NTB-2 emulsion in the dark and exposure was then carried out for 3–6 months at 4°C. Subsequently the slides were developed and stained with haematoxylin–eosin. As previously described, the extensive extractions in various media during the fixation and embedding procedures will remove unbound radioactivity, leaving tissue-bound radioactivity detected by autoradiography (27).

Fig. 2. A typical microautoradiograph showing the distribution of radioactivity in a buccal mucosal tissue explant exposed to [5-^{3}H]NNK. A tissue specimen was exposed to 6 μM of this agent for 24 h and then processed as described in Materials and methods.

Results

The BEX medium was found to support maintenance of the histomorphology of buccal explant cultures for between 2 to 5 days (not shown). Moreover, incubation of the explants in BEX medium with up to 100 μM NNK caused no detectable morphological alterations after a 2 day period of culture (not shown). Microautoradiographic analysis of covalently bound radioactivity in explants that had been exposed to [5-^{3}H]NNK for 24 h, showed extensive labelling of the epithelium, but no labelling of the connective tissue (Figure 2). The basal, middle and superficial epithelial cell layers were labelled to a similar extent, with silver grains localized both over the nuclei and the cytoplasm of the cells. Although the intensity of labelling varied among different donors, a similar and uniform distribution was observed in the various cell layers of the epithelium (not shown).

The metabolism of [5-^{3}H]NNK in both explants and epithelial cell cultures occurred by 3 major pathways, i.e. carbonyl reduction, pyridine *N*-oxidation and α-hydroxylation, and yielded the metabolites Nos 2–8 (Figure 1, Table I). At the concentrations (6 and 100 μM) chosen, to study metabolism by tissue specimens and cell cultures from various donors, formation of NNAL accounted for ~95% of the total metabolism (Table I). The formation of NNAL occurred at considerably faster rates at 100 μM than at 6 μM in both experimental systems. For the other metabolites, a marked increase in the rate of formation was only apparent in the cell cultures incubated with 100 μM NNK. The metabolites formed by α-hydroxylation and *N*-oxidation varied from undetectable amounts to levels corresponding to ~2% of the total metabolism. Overall, the seven identified metabolites accounted for 99–100% of the total metabolism in both experimental systems. The rate of total metabolism of NNK in secondary cultures (passage 1) of oral epithelial cells was 3- and 12-fold greater compared to explant cultures incubated with 6 μM and 100 μM NNK respectively (Table I). At the lower NNK concentration, the ratio of ketoacid to ketoalcohol ranged from 0.7 to 1.0 in cultured cells and explants. However, this ratio increased from 0.7 to 4.3 in cell cultures incubated with 100 μM NNK, and a similar, albeit less pronounced, result was observed in explants (Table I).

Comparison of the metabolism rates related to carbonyl

Table I. Metabolism of NNK in explants and cell cultures of human buccal mucosa obtained from various individuals[a]

No. of cases	NNK (μM)	Metabolic activity (nmol/mg/h)[c]	Distribution of identified metabolites[b] pmol/mg/h (%)						
			NNAL (2)[d]	NNK N-oxide (3)	NNAL N-oxide (4)	ketoalcohol (5)	ketoacid (6)	hydroxy acid (7)	diol (8)
Explants									
6	6	0.6 ± 0.3	556 ± 60 (94.8)	2.2 ± 0.8 (0.7)	3.5 ± 1.3 (1.5)	1.4 ± 0.5 (0.2)	1.4 ± 0.5 (0.5)	7.7 ± 2.9 (1.8)	1.3 ± 0.54 (0.6)
3	100	1.3 ± 0.9	1320 ± 272 (99.5)	0.1 ± 0.1 (0.2)	0.4 ± 0.3 (0.1)	1.1 ± 0.5 (0.1)	2.9 ± 2.2 (0.3)	1.6 ± 0.6 (0.1)	0.9 ± 0.5 (0.1)
Cells									
4	6	2.3 ± 1.4	2229 ± 1287 (94.7)	10.8 ± 6.2 (1.2)	3.5 ± 2.0 (0.4)	46.2 ± 26.7 (0.8)	29.6 ± 17.1 (2.1)	5.2 ± 3.0 (0.4)	7.6 ± 4.4 (0.5)
4	100	23.9 ± 13.8	23 151 ± 13 366 (96.2)	88.7 ± 51.2 (0.8)	57.5 ± 33.2 (0.2)	62.2 ± 36.5 (0.3)	416.1 ± 240.2 (2.0)	43.2 ± 24.9 (0.2)	25.2 ± 14.5 (0.1)

[a]The data in cells are from cultures in first passage, obtained as described in Materials and methods.
[b]Expressed as mean ± SEM.
[c]Explant cultures were incubated with NNK for 24 h and cell cultures for 1 h as described in Materials and methods.
[d]The figure corresponds to the number given to each identified metabolite in Figure 1.

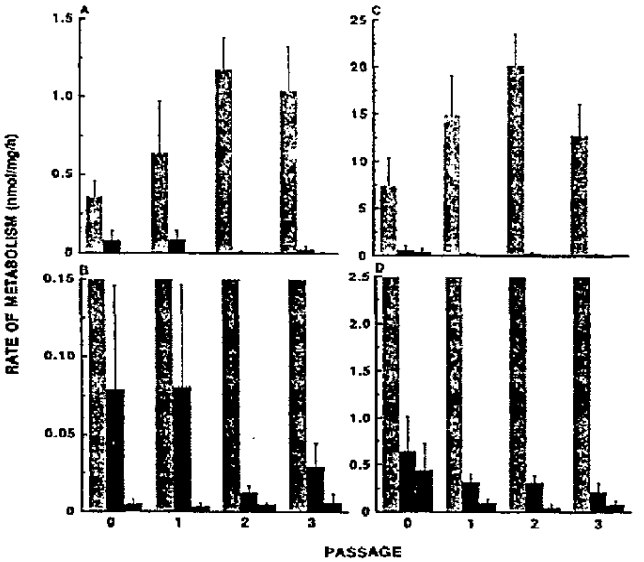

Fig. 3. Metabolism of NNK in cultured human buccal epithelial cells at various passages. Cells were exposed to 6 μM (A and B) and 100 μM (C and D) NNK respectively. B and D show a 10-fold enlargement of scales from A and C respectively, to facilitate the data interpretation of the α-hydroxylation and pyridine *N*-oxidation reactions. (▨) Carbonyl reduction, (■) α-carbon hydroxylation and (■) pyridine *N*-oxidation.

Table II. Covalent binding of NNK to protein in cultured human buccal explants and epithelial cells[a]

System	No. of cases	NNK (mM)	Protein adducts (pmol/mg)[b]
Explants	6	6	7.0 ± 4.9
	3	100	2.5 ± 1.2
Cells	5	6	1443.5 ± 509.6
	7	100	9580.2 ± 2781.4[c]

[a]For experimental details, see Materials and methods.
[b]Data expressed as mean ± SEM.
[c]This result is significantly different ($P < 0.01$) from the result obtained with the lower NNK concentration in this experimental system.

reduction, α-hydroxylation and pyridine *N*-oxidation of NNK in buccal epithelial cell cultures at various passages is shown in Figure 3. Cells from primary up to the third passage, maintained measurable metabolic activity for each of these pathways. At all passages, the rate of total metabolism was more than one magnitude greater at 100 μM than at 6 μM. In general, the primary and early transfer cultures seemed to be more proficient in α-hydroxylation and *N*-oxidation reactions than in the subsequent passages, in particular when examining the metabolism at the higher concentration. In contrast, the rate of reduction initially increased with each transfer, and was the highest in the second cell passage at both concentrations.

The formation of covalently bound protein adducts was detected in explants and cell cultures incubated with NNK at 6 and 100 μM (Table II). More than 100- and 1000-fold higher levels of protein adducts were detected in cell cultures as compared with explants at the respective concentrations. In cells, adduct formation increased significantly with the concentration and was almost 7-fold higher at 100 μM than at 6 μM NNK. In contrast, the amounts of adducts in explants at the respective NNK concentrations did not significantly differ.

The effect of NNK and its precursor alkaloid nicotine on CFE

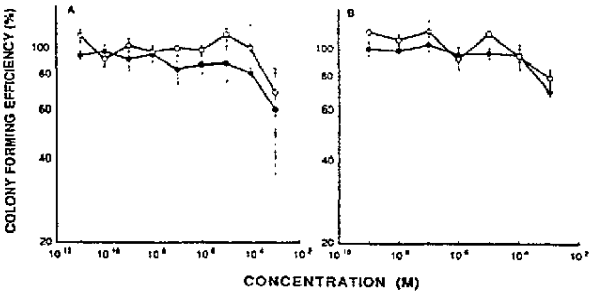

Fig. 4. Effect of nicotine and NNK on the colony forming efficiency of human normal and tumorous buccal epithelial cells. (A) nicotine; (B) NNK; normal epithelial cells (●); SqCC/Y1 cells (○).

of normal buccal epithelial cells and a buccal carcinoma cell line (SqCC/Y1) was determined (Figure 4). The rationale for these experiments was to investigate if, and at what concentration, these agents would induce cytotoxicity, or alternatively, would possibly stimulate growth, as has been shown in certain human lung cancer cell lines and mouse primary oral epithelial cells (28–32). Neither positive nor negative effects of these agents on CFE were detected in the nM to 100 μM concentration range. However, a concentration of 1 mM NNK significantly decreased the CFE of normal cells.

Discussion

Comparative studies of human tissues in explant culture have demonstrated the ability of many cancer-prone organs to metabolize various classes of chemical carcinogens, including *N*-nitroso compounds (14,33,34). Of all tobacco-specific *N*-nitrosamines, NNK is one of the most abundant, and also believed to be the most potent carcinogen (2). In the present study, incubation of buccal tissue explants and epithelial cell cultures for time periods previously used in similar studies of other tissues and cell types (11,12,14,15,33–36), clearly demonstrated the ability of human buccal mucosa to metabolize NNK by three major pathways (Figure 1). Reduction of NNK to NNAL was by far the major route of metabolism in both explant and cell cultures. This reaction is also the major metabolic route in laboratory animals and in many isolated experimental systems, including the rat oral mucosa in explant culture (11,12,14,15,36). The actual rate of NNAL formation in various experimental systems may be underestimated, because this metabolite becomes conjugated with glucuronic acid to a significant extent *in vivo* (37). However, the almost complete recovery of NNK-related radioactivity in the metabolism experiments, including those made with freshly obtained tissue, suggests that the rates of glucuronidation were minimal within human buccal epithelium. A lack of glucuronidation, which serves as a detoxification pathway for NNAL, may have a profound influence on the carcinogenic potency of NNK in the buccal mucosa, since accumulation of NNAL would result in additional substrate for α-hydroxylation pathways which produce intermediates capable of reacting with cellular macromolecules.

In the experimental systems utilized in the present study, the NNK metabolites originating from the two oxidative pathways accounted for ~5% or less of the total metabolism. Earlier studies with microsomes of human liver and rodent tissues demonstrated that oxidative NNK metabolism involves several isozymes in the cytochrome P450 family (38–40). As a result, *N*-oxidation and α-hydroxylation reactions are considered as deactivating and activating pathways respectively (1,15). The

formation of ketoalcohol, ketoacid, hydroxyacid and diol metabolites, which constitute an index of activation, in the buccal mucosal epithelium indicates that cytochrome P450 isozymes capable of metabolizing both NNK and NNAL to reactive intermediates are present in this human tissue. This is in agreement with previous studies showing that explant and epithelial cell cultures of rat oral mucosa activate procarcinogens including tobacco-specific *N*-nitrosamines (11,12,35). The fact that ketoacid formation predominated over ketoalcohol production at the higher substrate concentration of NNK utilized in the present study, could indicate that multiple cytochrome P450 isozymes with different K_m- and V_{max}-values are involved in α-hydroxylation of NNK at the *N*-methyl and methylene carbons in buccal mucosa. However, this possibility involves a dimension of uncertainty, since the contribution of the two pathways can not be clearly distinguished when measuring metabolites produced in tissue culture (11). Moreover, the presence of enzymes other than cytochrome P450 isozymes capable of metabolizing NNK should also be considered and subject to further studies.

Human buccal epithelial cells were shown to metabolize NNK in both primary culture and in several subsequent passages. The tendency that cells in primary culture exhibit higher activity than those in subsequent transfers for the α-hydroxylation and *N*-oxidation reactions, is in agreement with the fact that culturing cells commonly results in a decrease in cytochrome P450 content and loss of oxidative metabolism (41). In contrast, the cellular activity for carbonyl reduction increased with each passage up to the second transfer. Because buccal epithelial cell cultures exhibit their highest colony forming efficiency in these stages (22), carbonyl reduction seems to correlate with the proliferative potential. Interestingly, the reductase activity involved in this reaction is expressed early in rodent fetal development (42). Since the physiological function and endogenous substrate specificity of this enzymatic activity are unknown, further studies would be needed to explore the possible importance of these correlations.

The formation of macromolecular adducts, in particular the O^6-methylguanine adduct in DNA, following NNK metabolism in various target tissues and cell types has generally correlated with tumor origin and incidence (9,16,36,43). Support for the hypothesis that metabolic activation of [5-^{3}H]NNK results in the formation of adducts in human buccal mucosa is given from extensive covalent association of radioactivity in both tissue and cells. The autoradiographic visualization of explants, and protein extraction of both explant and cell cultures, demonstrate this finding. In explants, the lack of radioactivity in the connective tissue contrasted the uniform labelling of the epithelium. These results indicate that metabolic activation through α-hydroxylation reactions occurs primarily in the epithelium, and at similar rates in its various cell layers. Measurements of protein adducts in [5-^{3}H]NNK-exposed explant and epithelial cell cultures showed markedly higher binding levels in the cells. As evident from the autoradiographic analysis of tissue-bound radioactivity, the lower activity in tissue specimens may partly depend on dilution of epithelial protein with that from the major mass of metabolically inactive connective tissue. Alternatively, a facilitated uptake of NNK in cultured cells would possibly increase its metabolism.

The present study did not examine the formation of alkyl or pyridyloxobutyl adducts in DNA. Comparison of the results from the present study with those in NNK-exposed explant cultures of rat oral mucosa, in which the N7- but not the O^6-methylguanine adduct was detected (11), indicate that the ketoacid metabolite was formed at more than a 10-fold higher rate in the rat as compared to the human tissue. The O^6-methylguanine adduct is detected in certain cell types from rat lungs (36), in which the rates of ketoacid formation are also more than 10-fold higher than in human buccal cells. Therefore, the extent of DNA methylation in buccal cells would be expected to be considerably lower than in the above systems. It is unclear why protein binding did not increase as a function of concentration in buccal explant cultures, although the higher binding value at 6 μM agrees with the observation that higher total amounts of metabolites formed by α-hydroxylation were found at 6 μM than at 100 μM. Histologic examination of tissue sections indicated no toxicity following 48 h incubation with 100 μM NNK.

Growth of certain human lung cancer cells and mouse oral epithelial cells is stimulated by NNK (28,29,32). Moreover, nicotine may stimulate the growth directly or indirectly in lung cancer cell lines, in particular those of neuroendocrine origin, by suppressing the growth-inhibitory effect of other endogenous or exogenous agents, e.g. opioids (29–31). On the basis of these observations, nicotinic acetylcholine receptor-mediated actions have been hypothesized to constitute a growth-regulatory pathway that may become desensitized during carcinogenesis (30). In this regard, the reproductive ability measured as CFE of both normal cells and a tumorous buccal epithelial line at the similar serum-free conditions was not significantly altered by NNK or nicotine over a wide range of concentrations, including those which stimulated growth in the aforementioned systems. Therefore, the results indicate that these agents do not stimulate cell proliferation in human buccal epithelium. The decreased CFE at 1 mM of these agents showed that the NNK metabolism experiments in this study were performed at least one order of magnitude below a concentration that causes cytotoxicity.

In summary, the usefulness of *in vitro* methods for studying metabolism and macromolecular interactions of a potent tobacco carcinogen has now been demonstrated in a possible target tissue of human origin. The epithelium of human buccal mucosa, as an intact structure in tissue explants or as cultured cells, activate NNK to alkylating intermediates associated with mutagenicity and alkylation of plasma protein and DNA from respiratory tissues in smokers (2,6,9,44,45), supporting the hypothesis that NNK is important in the etiology of oral cancer in (smokeless) tobacco users. Buccal explants, and normal and transformed buccal epithelial cells at defined culture conditions may be useful experimental systems in future studies aimed at characterizing the protein and DNA adducts of tobacco-specific *N*-nitrosamines possibly involved in oral cancer, and the potential of such agents to alter cellular growth and differentiation.

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